

Higher plant transformation: principles and molecular tools

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ABSTRACT In higher plants, genetic transformation, which is part of the toolbox for the study of living organisms, had been reported only 30 years ago, boosting basic plant biology research, generating superior crops, and leading to the new discipline of plant biotechnology. Here, we review its principles and the corresponding molecular tools. *In vitro* regeneration, through somatic embryogenesis or organogenesis, is discussed because they are prerequisites for the subsequent *Agrobacterium tumefaciens*-mediated transferred (T)-DNA or direct DNA transfer methods to produce transgenic plants. Important molecular components of the T-DNA are examined, such as selectable marker genes that allow the selection of transformed cells in tissue cultures and are used to follow the gene of interest in the next generations, and reporter genes that have been developed to visualize promoter activities, protein localizations, and protein-protein interactions. Genes of interest are assembled with promoters and termination signals in *Escherichia coli* by means of GATEWAY-derived binary vectors that represent the current versatile cloning tools. Finally, future promising developments in transgene technology are considered.

KEY WORDS: *Agrobacterium tumefaciens*, T-DNA, transgene, plant transformation, somatic embryogenesis, organogenesis

Shoot regeneration in tissue culture

Genetic transformation usually involves DNA delivery to explants and subsequent tissue culture in which transformed cells are selected and induced either to form transgenic callus, shoots, roots, or somatic embryos. Hence, the tissue culture-induced regeneration capacity of a plant genotype is crucial for a successful genetic transformation. Indeed, recalcitrance to *in vitro* regeneration prevents genetic transformation in a large number of plant species or varieties. *In vitro* shoot regeneration competence has a genetic basis because it can be introgressed from a highly regenerative into a recalcitrant genotype (Koornneef *et al.*, 1993; Anami *et al.*, 2010). Therefore, identification of genes promoting or inhibiting the tissue culture-induced regeneration capacity will help to broaden the range of plant species for genetic transformation. Tissue culture regeneration occurs through organogenesis or somatic embryogenesis, which are discussed below and are schematically presented in Fig. 1.

Somatic embryogenesis

Somatic embryos develop from undifferentiated somatic cells in cultures and are morphologically and developmentally very similar to zygotic embryos, which are formed upon fertilization and surrounded by maternal tissue. Somatic embryogenesis was first demonstrated in embryogenic carrot (*Daucus carota*) liquid cultures that differentiated into somatic embryos at high frequency when diluted, sieved for the embryogenic subpopulation, and transferred to a medium lacking the synthetic hormone 2,4-dichloro-phenoxyacetic acid (2,4-D) (Steward *et al.*, 1958) (Fig. 1A). This embryogenic suspension culture system has been used as a model to study the regulatory genetic program of plant zygotic embryogenesis (Zimmerman, 1993). Indeed, all stages of the embryo formation, such as globular, heart, and torpedo, can be distinguished in the

Abbreviations used in this paper: GFP, green fluorescent protein; GUS, β -glucuronidase; T-DNA, transferred DNA; 2,4-D, 2,4-dichloro-phenoxyacetic acid; TALEN, transcription activator-like effector nuclease; *vir*, virulence; ZFN, zinc finger nuclease.

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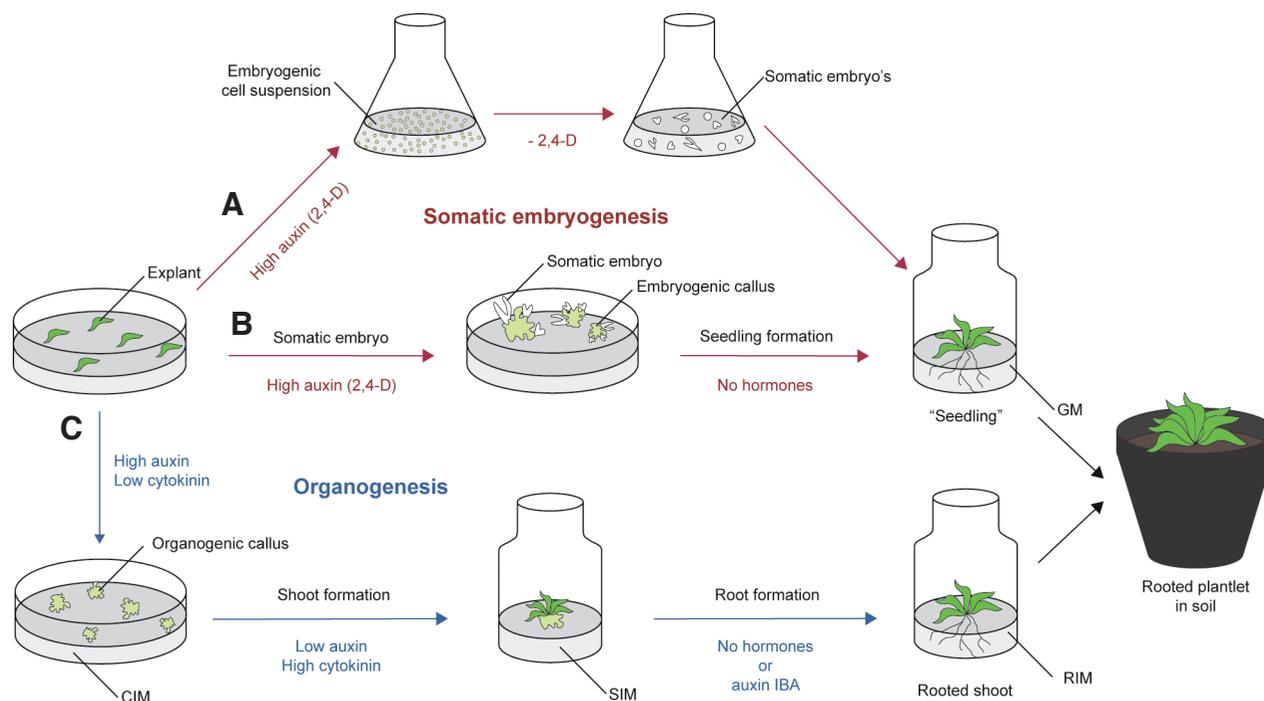


Fig. 1. Somatic embryogenesis versus shoot regeneration. (A) Somatic embryogenesis through embryogenic cell suspension cultures. **(B)** Somatic embryogenesis through embryogenic callus cultures. **(C)** Shoot regeneration through organogenesis.

embryogenic cultures and, upon removal of the exogenous auxin, the induction of the gene expression programs is comparable to that of the zygotic embryogenesis (Borkird *et al.*, 1986). The somatic embryogenesis receptor-like kinase SERK1 marks somatic plant cells that are competent to form somatic embryos in the carrot cultures (Schmidt *et al.*, 1997). Endogenous abscisic acid signaling is essential for carrot cells to acquire the embryogenic competence and for zygotic embryogenesis (Parcy *et al.*, 1994; Kikuchi *et al.*, 2006). Transcription factors expressed during embryogenesis, such as the ABSCISIC ACID-INSENSITIVE3/LEAFY COTYLEDON1/FUSCA3, are specific markers for embryogenesis that play a crucial role in the embryogenic process as demonstrated by knockout or overexpression lines (Lotan *et al.*, 1998).

Today, many plant species of agronomic and horticultural importance are regenerated in tissue cultures through somatic embryogenesis, among which the cereals (Shrawat and Lörz, 2006). Somatic embryogenesis in crop plants requires high concentrations of auxin, 2,4-D or dicamba to promote the vegetative-to-embryogenic transition in a small subset of cultured cells that are then committed to form somatic embryos. The somatic embryo is a bipolar structure with shoot apical meristem and radicle in contrast to the monopolar shoot structure originating through organogenesis. The embryo originates from a single cell and has no vascular connection with the maternal callus tissue or the cultured explant. Hence, somatic embryos are clonal unlike shoots regenerated by organogenesis that usually arise from several individual cells and might be chimeric, i.e. consisting of a mixture of independently transformed cells. Furthermore, induction of somatic embryogenesis requires a single hormonal signal to trigger a bipolar structure capable of forming a complete plant consisting of root and shoot and resembling a “seedling” (Fig. 1B). In organogenesis, shoots induced from organogenic

callus are removed from the callus to form roots on hormone-free or “rooting” medium (Fig. 1C).

Organogenesis

According to the “classical theory”, *in vitro* organogenesis refers to organ formation of *de novo* origin, involving phytohormone perception, dedifferentiation of differentiated cells into callus, acquisition of organogenic competence, re-entry of quiescent cells into the cell cycle, and organization of cell division to form specific organ primordia and meristems (Sugiyama, 1999). The pioneering work of Skoog and Miller (1957) and Christianson and Warnick (1983) established that auxin and cytokinin are the predominant growth regulators of tissue culture organogenesis. Shoot regeneration in tissue cultures usually needs a two-step hormone treatment (Fig. 1C). A high auxin/cytokinin ratio in the medium induces organogenic callus from an explant and, subsequently, a high cytokinin/auxin ratio triggers shoot formation. Such shoots originate typically from monopolar and callus-derived organ primordia, develop into shoots with leaves, and form roots upon transfer to medium without hormones or containing the “rooting” auxin isobutyric acid. Organogenic callus cultures with subsequent shoot organogenesis have been established in many species by means of numerous types of explants, including tobacco (*Nicotiana tabacum* L.) protoplasts (Nagy and Maliga, 1976), *Arabidopsis thaliana* root explants (Valvekens *et al.*, 1988) and leaf explants. A number of gene regulatory circuits important for dedifferentiation, redifferentiation, and adventitious meristem organization during *in vitro* regeneration of plants have been identified of which their components might be exploited as new tools to improve the plant regeneration efficiency (Duclercq *et al.*, 2011). Organogenic callus induction on auxin-containing medium corresponds with the competence (cell dedifferentiation) acquired by tissues to respond to hormonal signals and to upregulate

late AUXIN/INDOLE-3-ACETIC ACID genes. Shoot induction on cytokinin-containing medium coincides with the upregulation of the shoot apical meristem genes *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*) (Che *et al.*, 2006; Bao *et al.*, 2009), that might be applied in the future to enhance *in vitro* propagation as demonstrated by the overexpression of the *SHOOTMERISTEMLESS* (*STM*) and *ZWILLE2* (*ZLL2*) genes in *Brassica napus* (canola) and *Arabidopsis* plants (Elhiti and Stasolla, 2012; Chatfield *et al.*, 2013).

In tomato (*Solanum lycopersicum*) and *Brassica rapa* (turnip), in which organogenic callus induction and plant regeneration are quantitatively controlled by several genes (Trujillo-Moya and Gisbert, 2012; Seo *et al.*, 2013), candidate genes in the genetic quantitative trait locus (QTL) intervals were *APETALA2* (*AP2*)-containing ethylene response factors (ERFs), homologous to the well characterized *Arabidopsis* gene *ENHANCER OF SHOOT REGENERATION1* (*ESR1*) (Banno *et al.*, 2001), MADS box genes related to adventitious shoot regeneration (Prakash and Kumar, 2002), and serine-threonine kinases of which the tomato shoot kinase1 *LESK1* is an *in vitro* organogenesis competence marker (Torelli *et al.*, 2004).

Recent literature on shoot regeneration in *Arabidopsis* shows the need to revisit the concept of cell dedifferentiation upon organogenic callus formation (Sugimoto *et al.*, 2011). Shoots originate from organogenic callus derived from specific pericycle-like cells surrounding the vasculature in roots, hypocotyls, or cotyledon explants (Che *et al.*, 2007; Atta *et al.*, 2009). The transcript profile of organogenic callus is strikingly similar to that of lateral roots (Che *et al.*, 2006), indicating that shoot regeneration occurs via a "root developmental pathway" (Sugimoto *et al.*, 2010). As pericycle cells are the progenitors of lateral roots in primary roots, they might be considered as "adult meristem cells" that differentiate into organogenic callus from which shoots develop upon hormonal stimuli in tissue cultures (Sugimoto *et al.*, 2011).

Inherent to tissue culture procedures is the so-called somaclonal variation that refers to mutations, chromosome rearrangements and multiplication in some of the regenerated shoots, hinting at induction of stress reactions and accumulation of genetic aberrations during the dedifferentiation/redifferentiation processes (Jiang *et al.*, 2011). In order to restrict somaclonal variation, tissue culture duration should be kept as short as possible, tissue culture-related "stress" should be reduced by addition of antioxidants and organic buffer to the medium, a low light intensity, and minimal concentrations of selectable agents and hormones.

Techniques for higher plant transformation

The role of transgenes in higher plants can be studied through both transient and stable

transformation methods. Stable transformants are generated by means of *Agrobacterium* infection or particle bombardment and contain a stably integrated transgene in their plant genome that segregates as a Mendelian trait and is inherited in subsequent generations (Fig. 2). In contrast, transient transformants are obtained by biolistic treatment or agroinfection of explants, such as onion (*Allium cepa*) epidermis or protoplasts by which gene construct expression, subcellular localization, or protein targeting can be

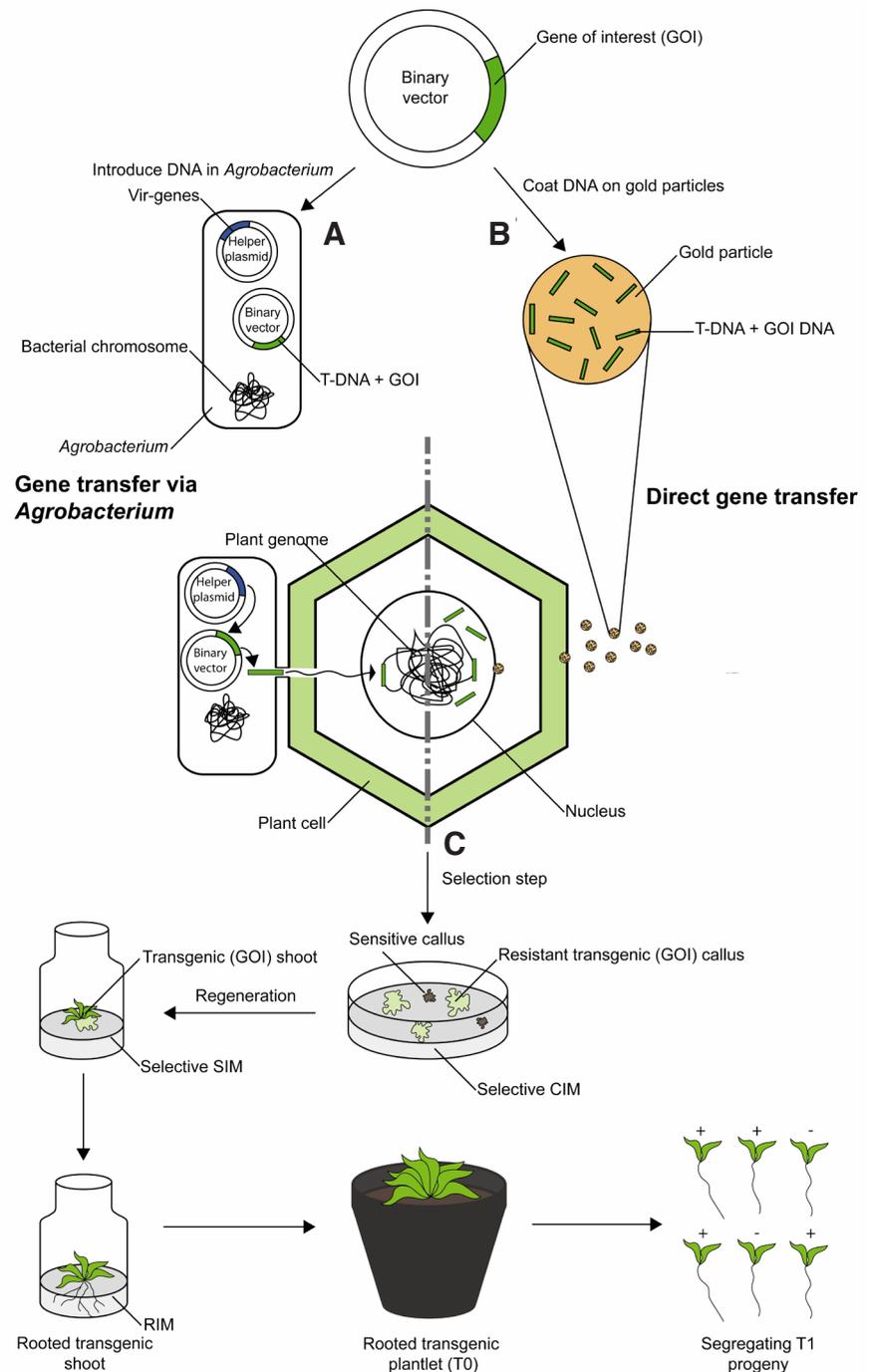


Fig. 2. Techniques for genetic transformation. (A) *Agrobacterium*-mediated T-DNA transfer. **(B)** Direct DNA transfer through particle bombardment or biolistics. **(C)** Regeneration and selection of transformed callus and shoots.

tested without integration of the transgene into the genome, thus circumventing *in vitro* shoot regeneration procedures (Sheen, 2001). Agroinfiltration of *Nicotiana benthamiana* leaves is the preferred method to investigate *in-vivo* protein-protein interactions by means of fluorescence resonance energy transfer and bimolecular fluorescence complementation technology in which proteins are fused with a fluorescent protein and their interaction is visualized by confocal microscopy (Yang et al., 2000; Boruc et al., 2010). Recently, transient transformation assays have been developed in cereals, such as rice (*Oryza sativa*) and maize (*Zea mays*). A high fluorescent marker gene expression has been demonstrated in bombarded leaf explants of maize that consisted of the basal-most 3 cm above the ligule of an approximately 50-cm growing adult leaf using different fluorescent protein tags and correct localization to the endoplasmic reticulum, the Golgi apparatus, and the plasma membrane was demonstrated (Kiriienko et al., 2012).

In stable transformants, transgenes are studied at the genetic, morphological, physiological, cell biological, and biochemical levels to gain insight into their function and might be the basis for translational or biotechnological research. Below, *Agrobacterium*-mediated transformation and direct gene transfer are discussed.

***Agrobacterium*-mediated transformation**

Agrobacterium tumefaciens-mediated transformation exploits the bacterium as the biological vehicle to transfer gene(s) of interest into the plant cell and involves a number of proteins derived from both the plant host and the bacterial pathogen (Gelvin, 2012). Naturally, the bacterium induces crown gall tumors on various plant species, including many agronomically important crops. During its infection, *Agrobacterium* replicates a single-stranded copy of the bacterial transferred (T)-DNA that is located on the tumor-inducing (Ti) plasmid and transfers it into the plant host cell where it subsequently integrates into the host genome. The wild-type T-DNA encodes several genes involved in auxin and cytokinin biosynthesis that are expressed in the infected plant cells, with cell proliferation and tumor formation as a consequence. With the help of other T-DNA-encoded genes, the tumors synthesize and secrete opines, amino acid derivatives that can be metabolized mainly by *Agrobacterium*. This unique infection strategy allows *Agrobacterium* to hijack the host cell machinery and turn it into its own "food factory". Although *Agrobacterium* mainly infects dicotyledonous plants in nature (De Cleene and De Ley, 1976), it can genetically transform a wide range of higher plant species under laboratory conditions and has become the transformation vehicle of choice for the genetic manipulation of most plant species (Tzfira and Citovsky, 2006).

The T-DNA was adapted for genetic engineering by removing the hormone and opine biosynthesis genes and by replacing them with a selectable marker gene and one or more genes of interest, flanked by the T-DNA left and right border sequences that delineate the T-DNA. The T-DNAs are engineered in *Escherichia coli* on a binary vector that is introduced into an *Agrobacterium* strain containing a T-DNA-less, so-called "helper", Ti plasmid harboring the virulence (*vir*) functions that are required for the bacterium-host recognition, the T-DNA replication in the bacterium, and the T-DNA transfer to and integration into the plant genome (Fig. 2A). *Agrobacterium* containing the engineered vectors are usually co-cultivated with specific explants from which transgenic

shoots or somatic embryos are regenerated and selected in tissue cultures (Fig. 2C). In *Arabidopsis*, floral buds can be submerged in a liquid *Agrobacterium* culture and transgenic plants can be selected from the seeds (Clough and Bent, 1998), a method that avoids tissue culture procedures.

Upon *Agrobacterium*-mediated transformation, usually intact, single, or tandemly arranged T-DNA copies in one or two loci are stably integrated into AT-rich regions of the nuclear plant genome with minimal rearrangement of the target site (Gheysen et al., 1991). At low frequency, T-DNAs are truncated at their left border and vector backbone DNA is integrated (Tzfira and Citovsky, 2006). The selectable marker and the gene of interest, located on the T-DNA, are inherited in subsequent generations and segregate according to Mendelian genetics (De Block et al., 1984; Horsch et al., 1984). *Agrobacterium*-mediated transformation is the method of choice for overexpression or down-regulation of genes of interest in functional basic research or for generation and commercialization of superior crops thanks to its rather precise replication through the T-DNA border sequences upon infection and the low-copy T-DNA insertion into the genome. The T-DNA has been used as mutagen itself or to introduce transposons for mutagenization into several plant species, resulting in mutagenized collections that are invaluable resources for gene identification and functional analysis (Alonso et al., 2003).

Direct gene transfer

In nature, the host range of plant species and genotypes that are competent for *Agrobacterium* infection is limited, indeed, competence is determined by bacterial and plant host genes the study of which might help to enlarge the natural *Agrobacterium* host range. To overcome competence barriers, "direct" gene transfer methods have been developed in which genes of interest are delivered directly into regenerable plant tissues. Gene transfer by high-velocity microprojectiles (biolistic or particle bombardment) is widely used and has expanded the range of species and genotypes for genetic engineering (O'Kennedy et al., 2011) (Fig. 2B). Metal particles coated with naked plasmid DNA containing the gene of interest are transported to the plant cells by means of an electric discharge or in a pressurized helium stream. Nearly all of the physical and chemical parameters (rupture pressure, DNA concentration, particle travel distances, and vacuum degree) can be adjusted to different tissues and species. One of the first successes was the transfer of foreign genes into intact maize cells of a Black Mexican Sweet cultivar suspension culture (Klein et al., 1988). Particle bombardment is frequently used for the transformation of cereals with immature embryos as explants (Shrawat and Lörz, 2006) and is the only effective method to transform chloroplasts in plants.

However high-copy numbers and extensive rearrangements of the foreign DNA have frequently been reported in plants stably transformed with direct gene transfer methods. The integration of too many copies of the same gene within the genome normally results in gene silencing. In addition, sequences of the introduced gene have been found to be truncated, making the transgene analysis difficult and undesirable. Only DNA fragments of less than 10 kb in size can be transferred by the biolistic technology because large fragments get destroyed during the bombardment or adhere poorly to the metal particle, with messy DNA integration events as a consequence (Shou et al., 2004).

Selectable marker genes

Selectable marker genes are used to identify cells that have incorporated the T-DNA with the marker gene and the gene of interest upon genetic transformation of explants as well as to monitor the transformed individuals in subsequent generations. Upon infection or biolistic treatment of an explant, only a very small proportion of the cells is transformed; thus, the probability of recovering transformed lines without a selection system is very low. The most frequently used selectable markers include antibiotic resistance genes, such as neomycin and hygromycin phosphotransferases, herbicide resistance genes, such as phosphinothricin *N*-acetyltransferase and aceto lactate synthase, and metabolism-related genes, such as phosphomannose isomerase. These marker genes have been adopted for widespread use because of their efficiency and general applicability to a wide range of species and tissue culture systems (Table 1). Additional selectable metabolism-related markers, such as xylose isomerase, trehalose-6P-synthase, and protoporphyrinogen oxidase, are less frequently used (Miki and McHugh, 2004). To function in a variety of cell types, selectable marker genes are constructed as chimeric genes, including regulatory sequences that ensure constitutive expression throughout the plant (Miki and McHugh, 2004).

Neomycin phosphotransferase II gene

Neomycin phosphotransferase II (NPTII), also known as bacterial amino-glycoside 3'-phosphotransferase II (APH [3']), is an enzyme encoded by the *nptII* or *neo* gene that had been isolated from the *E. coli* transposon Tn5. It confers resistance to its host cells against a wide range of amino-glycoside antibiotics, including kanamycin, neomycin, geneticin (G418), and paramomycin, by catalyzing the phosphorylation of the 3'-hydroxyl group of the amino-hexose portion of these aminoglycosides. In this manner, NPTII detoxifies the antibiotic, allowing transformed *nptII*-expressing plants to tolerate certain concentrations of the aminoglycosidic antibiotics, unlike the nontransformed plants that undergo bleaching and growth inhibition at similar concentrations. The *nptII* gene has been used as a selection marker for vectors in prokaryotic and eukaryotic cells. It is the most widely used selectable marker system for generating transgenic plants, especially in dicotyledonous plants, such as *Arabidopsis* (Miki and McHugh, 2004). For constitutive expression in plants, the coding region of the *nptII* gene has been fused to the 5'- and 3'-regulatory

sequences of the *Agrobacterium* T-DNA gene nopaline synthase (*nos*). This gene construct has been shown to be efficient in selection of tobacco cells on kanamycin or G418 (Herrera-Estrella *et al.*, 1983). Transformants sensitive and resistant to kanamycin and their progenies can be distinguished through different techniques, including seed germination assays on kanamycin-containing medium carried out to follow the *nptII* gene segregation in the progeny of primary transformants (De Block *et al.*, 1984) and callus induction tests (Van Lijsebettens *et al.*, 1991). Enzymatic *in vitro* assays that can detect the NPTII protein quantitatively or semiquantitatively are based on the transfer of the ³²P-labeled γ -phosphate group from ATP to kanamycin (Reiss *et al.*, 1984), whereas an enzyme-linked immunoabsorbent assay has been developed as well (Nagel *et al.*, 1992).

Hygromycin phosphotransferase gene

The hygromycin phosphotransferase (*hpt*) gene, also designated *aphIV*, was isolated from *E. coli* and encodes the enzyme hygromycin B phosphotransferase that confers resistance to the antibiotic hygromycin B (Waldron *et al.*, 1985), an aminocyclitol antibiotic that inhibits protein synthesis with a broad spectrum activity against prokaryotes and eukaryotes. The *hpt* gene has been modified for expression in plant cells by developing a chimeric gene consisting of the *nos* regulatory elements and the *E. coli*-derived *hpt* gene (van den Elzen *et al.*, 1985). Hygromycin is more toxic in plants than kanamycin; hence, the *hpt* selectable marker is used when *nptII* is not effective. Hygromycin resistance in transformed plants can be checked in several ways, including callus induction tests (Van Lijsebettens *et al.*, 1991), seed germination assays to score the segregation of the *hpt* gene in the transgenic plant progenies, and enzymatic assays (Datta *et al.*, 1990).

Bialaphos-resistant and phosphinothricin N-acetyltransferase genes

The bialaphos-resistant (*bar*) gene, isolated from *Streptomyces hygroscopicus* (Murakami *et al.*, 1986) and the *pat* gene, isolated from *Streptomyces viridochromogenes* (Wohlleben *et al.*, 1988), are 87 % homologous and code for the enzyme phosphinothricin *N*-acetyltransferase (PAT) that catalyzes the conversion of phosphinothricin (PPT) into a nontoxic acetylated form (Thompson *et al.*, 1987). PPT, also designated glufosinate ammonium, is an active ingredient of several broad-spectrum herbicide formula-

TABLE 1

FREQUENTLY USED SELECTABLE MARKER AND REPORTER GENES IN PLANT TRANSGENIC RESEARCH

Gene	Substrate – Enzyme	Origin	Effect	Reference
<i>NPT II</i>	Neomycin, kanamycin, geneticin (G418) – paramomycin neomycin phosphotransferases II	<i>Escherichia coli</i>	Inactivates a number of aminoglycoside antibiotics by phosphorylation	Fraley <i>et al.</i> (1983)
<i>HPT</i>	Hygromycin – hygromycin phosphotransferase	<i>Escherichia coli</i>	Inhibitor of protein synthesis	Waldron <i>et al.</i> (1985)
<i>BAR, PAT</i>	Phosphinothricin (PPT) – phosphinothricin acetyltransferase	<i>Streptomyces hygroscopicus</i> , <i>Streptomyces viridochromogenes</i>	Acetylation of PPT a competitive inhibitor of glutamine synthetase	De Block <i>et al.</i> (1989), Wohlleben <i>et al.</i> (1988)
<i>ALS</i>	Sulfonylureas and imidazolinones – acetolactate synthase	Mutated form: <i>Arabidopsis thaliana</i> , <i>Oryza sativa</i> , <i>Zea mays</i> , <i>Malus domestica</i>	Mutant ALS enzymes insensitive to herbicides	Olszewski <i>et al.</i> (1988)
<i>manA</i>	D-mannose – phosphomannose isomerase	<i>Escherichia coli</i>	Mannose used as carbon source	Joersbo <i>et al.</i> (1998)
<i>EGFP</i>	None – enhanced green fluorescent protein	<i>Victoria aequorea</i> (modified from GFP)	Fluorescence	Yang <i>et al.</i> (1996)
<i>GUS</i>	β -glucuronides – β -glucuronidase	<i>Escherichia coli</i>	Hydrolyzation of β -glucuronides	Jefferson <i>et al.</i> (1987)
<i>LUC</i>	Luciferin – luciferase	<i>Photinus pyralis</i>	Oxidative decarboxylation of luciferin	Ow <i>et al.</i> (1986)
<i>R & CI</i>	None – R and CI anthocyanin transcriptional regulators	<i>Zea mays</i>	Anthocyanin accumulation in cell vacuoles	Ludwig <i>et al.</i> (1990), Lloyd <i>et al.</i> (1992)

tions, such as Basta™, Ignite™, and Liberty™. It is an L-glutamic acid analog and a competitive inhibitor of glutamine synthetase, the only enzyme that can catalyze ammonium assimilation into glutamic acid in plants. Inhibition of glutamine synthetase results in the rapid accumulation of ammonia and, eventually, plant cell death. The PAT enzyme catalyzes the acetylation of the free amino group of PPT, making it unable to bind to and inactivate glutamine synthetase. To engineer herbicide resistance in plants, the *bar* gene was placed under the control of a 35S cauliflower mosaic virus (CaMV) promoter and transgenic tobacco plants were resistant to high doses of PPT and bialaphos (De Block *et al.*, 1987). Today, it is frequently used as a selectable marker in cereals, such as maize, wheat (*Triticum aestivum*), rice, and barley (*Hordeum vulgare*) (Shrawat and Lörz, 2006). Several assays have been developed for the *bar/pat* marker gene activity in transgenic progenies, such as germination on PPT-containing medium, spraying plants, or painting leaves with the herbicide. Upon PPT treatment, ammonium accumulation in the medium can be determined with a colorimetric assay, specifically of nontransgenic seedlings that cannot assimilate ammonium due to glutamine synthase inhibition in contrast to transgenic seedlings that assimilate ammonium (De Block *et al.*, 1995). PAT proteins can be detected simply and quickly with the PAT assay kit (AgraStrip®LL Strip test kit; Romer Labs®, Union, MO, USA) that is based on a double-antibody sandwich principle.

Mutated acetolactate synthase genes

Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathway of the branched-chain amino acids isoleucine, leucine, and valine. The plant ALS gene is a target for several classes of herbicides, including sulfonylurea, imidazolinone, and pyrimidinyl carboxyl. Naturally occurring mutations in ALS confer herbicide resistance to a number of plant species (Chang and Duggleby, 1998). Single or double amino acid substitutions can induce herbicide resistance and have been utilized as effective selectable markers in homologous or heterologous plant species. For instance, the *Arabidopsis als* mutant has been applied in the selection of transgenic tobacco plants growing in a sulfonylurea herbicide-containing culture (Olszewski *et al.*, 1988). Different point mutations in the ALS gene that alter conserved amino acids have been isolated in rice and used as selectable markers into rice, wheat, and soybean (*Glycine max*) (Rosellini, 2011). The *als* mutant very efficiently selected transgenic maize in embryogenic cultures (Fromm *et al.*, 1990); similarly, the *als* mutants generated by site-specific mutagenesis were used successfully as selectable markers in tobacco and apple (*Malus domestica*) transformation (Yao *et al.*, 2013). The use of mutant plant ALS genes as selectable markers for transgenic plants is gaining importance because they occur in all plants and, thus, are expected to be publicly acceptable, not prompting food safety concerns as in the case of bacteria-derived selection genes.

Phosphomannose isomerase gene

The phosphomannose isomerase gene (*pmi*) gene isolated from *E. coli* (also designated *manA* gene) encodes the enzyme phosphomannose isomerase (PMI) that catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate. Mannose-6-phosphate is obtained from mannose through a hexokinase phosphorylation activity; unlike fructose-6-phosphate,

most plants cannot use it as a natural carbon source. Transgenic PMI-producing plants have a metabolic advantage over the non-transformed plants, because they are able to utilize mannose as a carbon source by converting mannose-6-phosphate to fructose-6-phosphate and can survive on a mannose-containing medium as the only or major carbon source. In nontransformed plants, mannose-6-phosphate accumulation inhibits glycolysis and leads to an arrest in cell growth and development. Although PMI is widely distributed in nature and found across kingdoms, it is absent in many plants, with the exception of soybean and other leguminous plants (Goldsworthy and Street, 1965). The PMI selection system with the *E. coli manA* gene under the control of the 35S promoter has been reported to be very efficient. Indeed, transformation frequencies in sugar beet (*Beta vulgaris*) increased 10-fold when compared to the kanamycin selection (Joersbo *et al.*, 1998). Similarly, the *E. coli manA* gene under the control of the maize ubiquitin promoter was reported to perform well in dicotyledonous and monocotyledonous plants (Reed *et al.*, 2001). PMI expression assays have been applied to identify transgenic events and to sort them through segregating populations, such as a modified chlorophenol red assay (Kramer *et al.*, 1993), in which the medium changed from red to yellow in tissues that could not metabolize mannose, and a seedling germination assay, in which the germination of nontransgenic seedlings was completely inhibited when mannose was introduced into the medium (Reed *et al.*, 2001).

Reporter genes

Reporter genes, also called screenable or scorable markers, are genes that code for proteins that can be detected directly or catalyze specific reactions with easily detectable products. They are particularly useful for promoter activity analysis, protein localization, and/or interaction studies. An ideal genetic reporter system should be *in situ* detectable, sensitive, quantitative, rapid, reproducible, safe, and with low or without endogenous background activity. The β -glucuronidase (GUS), luciferase (LUC), enhanced green fluorescent protein (EGFP) and anthocyanin are the most commonly used reporter genes in plant research (Table 1).

Green fluorescent protein

The green fluorescent protein (GFP) is a photoprotein cloned from the jelly fish *Aequorea victoria* (Shimomura *et al.*, 1962; Prasher *et al.*, 1992). It is a very stable protein that autofluoresces in the presence of UV or blue light illumination and does not require an external substrate. Niedz *et al.* (1995) were the first to show that the wild-type *Aequorea* GFP could be visualized in plant cells as an *in vivo* reporter of plant gene expression. Although wild type GFP was used successfully in plant cell and tissue expression studies, it had some disadvantages, such as aberrant splicing in plants and formation of cytotoxic and nonfunctional aggregates. Effective expression in whole plants was achieved upon modification of the GFP-coding sequence (Haseloff *et al.*, 1997) that improved fluorescence intensity and thermostability. GFP has a small molecular size, thus facilitating the construction of fusion proteins for subcellular protein localization or protein-protein interactions. GFP visualization is nondestructive and allows the direct imaging of gene products in living tissues in real time at the cellular level. GFP has an autofluorescence capacity and,

therefore, does not require addition of cofactors or exogenous substrates to produce light; moreover, it is very stable to heat, extreme pH, and chemical denaturants. Several GFP mutants have been developed through amino acid substitution, resulting in variants with altered excitation and emission spectra, such as variants with shifts to cyan, red, and yellow that are used for colocalization of specific proteins; for a detailed discussion, see Vo β *et al.* (2013). EGFP is one of these GFP variants that is commonly utilized because of its increased fluorescence intensity (Yang *et al.*, 1996).

β -Glucuronidase

β -Glucuronidase (GUS) is a bacterial enzyme encoded by the *E. coli uidA (gusA)* gene that occurs in microorganisms, vertebrates, and invertebrates, but not in most higher plants (Jefferson *et al.*, 1987). It catalyzes the hydrolysis of a wide variety of β -glucuronides, such as the chromogenic histochemical 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc), a colorless compound that is converted by the β -glucuronidase enzyme to an insoluble indigo blue product. It is used for *in situ* histochemical localizations of the β -glucuronidase activity in cells and tissues. The fluorogenic assay, in which β -glucuronidase cleaves the 4-methyl-umbelliferyl glucuronide (MUG) substrate into a blue fluorescent compound, quantifies the promoter strength (Cousens *et al.*, 2012). The GUS enzyme is very stable within plants and nontoxic when produced at high levels (Miki and McHugh, 2004), but the assays are destructive.

Luciferase

The luciferase (*luc*) reporter gene originates from the firefly *Photinus pyralis* and encodes the enzyme luciferase (LUC) that catalyzes the ATP-dependent oxidative decarboxylation of luciferin, leading to an excited form of oxyluciferin and to light emission. The flash of light is captured with a luminometer that measures the integrated light output. The total amount of light measured during a given time interval is proportional to the amount of luciferase activity in the sample. Typically, the light flash decays in seconds; enhanced light intensity and a more sustained light reaction were obtained when coenzyme A was added to the reaction, thus improving reactivity and reproducibility of the assay. Initially, the *in vivo* LUC activity was detected by spraying plant tissues with a luciferin substrate and squeezing it onto a film for exposure (Ow *et al.*, 1986); currently, the faint light from the reporter can be detected with specialized cameras. An advantage of the LUC reporter system is that it permits the nondestructive monitoring of the gene expression patterns, including circadian rhythms, in real time and with great accuracy (Millar *et al.*, 1992; Xu *et al.*, 2010). In addition, the luciferase assay is highly sensitive and results can be obtained within minutes. The limitations of the *in vivo* applications were overcome by the development of soluble luciferin forms that allow cell penetration. Thus, LUC has been used to study reporter gene activities *in vivo* in whole organisms, such as plants, as well as in single cells.

Anthocyanin

Anthocyanins are endogenous pigments that are responsible for the red, purple, and blue color in flowering plants, form a diverse family of aromatic flavonoid compounds, and play a role in protection against UV, defense response, and attraction of pol-

linators and seed dispersers. The biosynthesis of anthocyanins is controlled by a conserved triad of transcriptional regulators (an R2R3-MYB protein, a basic helix-loop-helix [bHLH], and a WD40 repeat protein) of which the overexpression increases the anthocyanin pigment accumulation that has been exploited to monitor both transient and stable gene expression in plants. Overexpression of one or more types of these regulatory genes led to cell-autonomous pigmentation in maize, *Arabidopsis*, and tobacco (Ludwig *et al.*, 1990; Lloyd *et al.*, 1992; Chu *et al.*, 2013). Anthocyanin accumulation is used as a visual marker in cereal transformation and transgenic seeds (Kawahigashi *et al.*, 2007; Gao *et al.*, 2011). As the anthocyanin reporter is nondestructive, does not require an exogenous substrate, and is not toxic, there are no related environmental and health concerns.

Promoters for chimeric gene construction

A promoter refers to the region in the genome sequence upstream of a gene transcription start site that controls the gene expression level and the kind of specificity, i.e. constitutive, inducible, tissue-specific, or developmentally regulated. In transgenic research, promoters are used to drive the expression of the selectable marker to select transformed callus and shoots during the transformation procedure, to follow the T-DNA segregation with the gene of interest in subsequent progenies, and to determine the expression level and specificity of the gene of interest in the progeny. Promoters either originate from heterologous species, which avoids gene silencing, or are cisgenic, which is considered similar to plants bred through conventional breeding methods and, therefore, is more acceptable to consumers.

Constitutive promoters

Constitutive promoters drive high gene expression levels in all cell types throughout the entire growth and development period and are used to overproduce proteins of interest to study their function in basic research or to generate superior plants or seeds for agronomical purposes. The *35S CaMV* gene (Odell *et al.*, 1985) confers a high transgenic expression in most cell types, except in pollen, is independent of environmental conditions, and is well characterized and active in various monocotyledonous and dicotyledonous plants (Benfey and Chua, 1990). The maize *Ubi-1* promoter is derived from the constitutively expressed ubiquitin gene (Christensen *et al.*, 1992) and is generally used in cereals (Shrawat and Lörz, 2006). Other strong constitutive promoters with a lot of potential in plant biotechnology have been identified in the rice actin genes *OsAct1* and *OsAct2* (McElroy *et al.*, 1990), in the *Brachypodium distachyon* elongation factor and ubiquitin genes *BdEF1 α* and *BdUBI10* (Coussens *et al.*, 2012; Karimi *et al.*, 2013), and in the switchgrass (*Panicum virgatum*) ubiquitin genes *PvUbi1* and *PvUbi2* (Mann *et al.*, 2011).

Organ-, tissue-, domain- or cell type-specific promoters

Specific promoters are utilized when the transgene expression is preferred at a specific site and/or specific time in development to generate specific phenotypes and avoid adverse effects on plant growth or yield. Several endosperm-specific promoters have been identified that express single or, even, multiple enzymes of biochemical pathways either to dissect the metabolic pathway or to improve the nutritional seed quality (Naqvi *et al.*, 2009; Cous-

sens *et al.*, 2012). Other promoters have been used for basic or biotechnological research, such as the TA29 tapetum-specific promoter that has been applied successfully for the generation of male sterility in canola (Mariani *et al.*, 1990). The *AP1* promoter of the A-type flower gene *AP1*, which is active in floral whorl one and two- silenced the *AP3* gene in whorl two and created a doubled sepaloid flower in *Arabidopsis* and canola (Byzova *et al.*, 2004) that avoided reflection of the bright-yellow canopy, captured more sun light, and increased yield.

Inducible promoters

Inducible promoters are specifically activated in response to external stimuli. In contrast to constitutive promoters, the fused transgenes can be expressed at a distinct developmental stage for a certain duration or in a specific tissue. Additionally, the promoters are inactive in the absence of inducers and, therefore, have no negative impact on plant development. The promoter activity can be induced by chemical factors, such as tetracycline, ethanol, steroids, copper ions, and herbicides, or by physical factors, such as heat, cold, and light. Promoters that respond to specific chemical compounds, not found naturally in the organism of interest, are of particular interest in genetic engineering because of the ease of manipulation. Some of the most commonly used chemically inducible promoters in plants (Padidam, 2003) are briefly described. Tetracyclines are particularly attractive as gene expression inducers, because they are small lipophilic compounds that enter easily into eukaryotic cells by passive diffusion and they have been routinely used in both human and veterinary medicine with negligible side effects. The tetracycline-inducible system consists of three main components: the transcriptional repressor, the tetracycline-responsive operator, and an antibiotic of the tetracycline family. The tetracycline-inducible system has been used successfully to produce valuable pharmaceutical or industrial proteins in plant cell suspension cultures (Bortesi *et al.*, 2012). In the steroid-inducible systems, heterologous proteins are fused to a receptor for glucocorticoid or estrogen and induced by steroids. The glucocorticoid receptor-based steroid-inducible system has significantly advanced the insight into the function of plant transcription factors that control plant developmental pathways (Lloyd *et al.*, 1994; Aoyama *et al.*, 1995). The ethanol-inducible gene expression system is derived from the filamentous fungus *Aspergillus nidulans* and consists of two elements: the alcohol-regulated transcription factor (ALCR) that binds the alcA-derived promoter that regulates the expression of the transgene (Roslan *et al.*, 2001). The ethanol-inducible system has been optimized for the production of proteins in plants (Dugdale *et al.*, 2013).

Databases for plant promoter sequences

Functional analysis of genes in transgenic plants often demands selection of promoters with appropriate activity patterns. Promoters commonly used in vectors are very limited and provide only little variation in gene expression patterns. Thus far, with an increasing number of sequenced plant genomes, it has become necessary to develop a robust computational method for detecting novel plant promoters. To date, a wide variety of programmes for predicting promoters are available, including PlantPAN (Chang *et al.*, 2008), GRASSIUS (Yilmaz *et al.*, 2008), PlantCARE (Lescot *et al.*, 2002) and TransGene Promoters (TGP) database (Smirnova *et al.*, 2012). However, promoters identified

by prediction programs need to be tested by means of reporter genes during plant development and under different stimuli to be applicable in transgenic research.

Vectors for higher plant transformation

Binary vectors

A binary vector system (Lee and Gelvin, 2008) consists of two plasmids: the helper plasmid that is constituted of the *Agrobacterium* Ti plasmid without T-DNA, but carries the *vir* genes that are necessary for the T-DNA transfer in the plant host genome and acts *in trans*, and the binary plasmid derived from the commonly used *E. coli* cloning vectors and carrying the gene of interest, flanked by 25 bp terminal repeats, designated the right and left T-DNA border sequences. The binary cloning vector is a standard molecular tool in the *Agrobacterium*-mediated transformation of higher plants, because it is easy to manipulate *in vitro* by recombinant DNA methods (Bevan, 1984). Cloning vectors can be assembled to facilitate fusion, overexpression, or downregulation of a variety of genes in plant cells. Their basic skeleton includes a gene of interest under a specific promoter in addition to a selectable and/or reporter gene. Superbinary vectors have additionally the *virB*, *virG*, and *virC* virulence genes of the supervirulent pTiBo542 plasmid, but require yet another intermediate cloning vector and cointegration step in *Agrobacterium*, hence complicating the cloning (Komori *et al.*, 2007). A good alternative strategy is the use of the supervirulent *Agrobacterium* strain EHA101 that contains the supervirulent *vir* genes of pTiBo542 in the helper plasmid (Hood *et al.*, 1986; Frame *et al.*, 2002; Coussens *et al.*, 2012).

GATEWAY vectors are a set of versatile and robust T-DNA binary vectors that enable quick and easy cloning and transfer of DNA fragments between vector backbones. The utilization of these vectors overcomes the cumbersome conventional cloning procedure, involving DNA restriction and ligation reactions, and the efforts to develop small vectors with unique restriction sites. The GATEWAY cloning technology takes advantage of the site-specific reversible recombination system of phage λ that enables rapid and efficient cloning and transfer of DNA fragments related to promoters, cDNA, or gDNA between different expression vectors (Hartley *et al.*, 2000). The DNA fragment is first captured in a GATEWAY donor vector (pDONR) through a site-specific recombination reaction resulting in a GATEWAY entry clone (pENTR). Subsequently, the DNA fragment can be recombined into many different GATEWAY destination vectors (pDEST), depending on the necessity for overexpression, silencing, and promoter analysis, and results in an expression clone (pEXPR). Several components are essential in the GATEWAY cloning procedure: the *att* sites, the *ccdB* gene, and clonase enzymes that recognize the *att* site. The GATEWAY *att* sites are phage-derived recombination sites that facilitate directional cloning and maintain orientations and reading frames of the DNA fragments. The *attB* sites that flank the DNA of interest recombine with *attP* sites in a donor vector yielding *attL* sites in a novel entry clone, a reaction catalyzed by the BP clonase enzyme. Similarly, the *attL* sites in the entry vector recombine with the *attR* sites of a destination vector to yield the *attB* sites in a novel expression clone. Directional cloning is enabled by ensuring that only specific sites recombine, for instance, *attB1* with *attP1* and not *attP2* (Hartley *et al.*, 2000). The BP clonase enzyme is composed of the phage integrase and the *E. coli* integration host factor, whereas the LR

clonase consists of the phage integrase, the *E. coli* integration host factor, and the phage excisionase. GATEWAY vectors can be selected and maintained by the use of *ccdB* gene and antibiotic selection markers. The *ccdB* gene is a negative counterselection marker encoding a protein that interferes with the DNA gyrase, thus inhibiting the *E. coli* growth. *ccdB* is present in the pDONR or pDEST vectors and is replaced by the DNA of interest upon recombination; hence, *E. coli* cells with the correctly recombined plasmids will survive, whereas cells with unreacted vectors or byproduct-containing *ccdB* will fail to grow. MultiSite GATEWAY is an extension of the GATEWAY technology, involving additional novel recombination sites with unique specificities to enable the simultaneous cloning of multiple fragments in a single highly efficient and specific *in vitro* LR clonase reaction. The fragments are cloned in an expression vector in a predefined order, orientation, and translation reading frame (Cheo *et al.*, 2004).

GATEWAY vectors for plant transformation

GATEWAY destination vectors have been developed to analyze the gene function through *Agrobacterium*-mediated transformation of dicotyledonous or monocotyledonous plants (Karimi *et al.*, 2007, 2013; Himmelbach *et al.*, 2007). A versatile set of GATEWAY-compatible destination vectors has been constructed to be used in monocotyledonous plants for improvement of transgenic crops either through transgene overexpression or interference RNA (RNAi)-mediated gene suppression (Mann *et al.*, 2012). Unique plant GATEWAY RNAi vectors for the functional analysis of the metabolic pathway in root tissues have been described (Muranaka, 2011). Functional elements built as GATEWAY entry clones, such as promoters, terminators, open reading frames, or diverse tags, can be recombined in a single step in the MultiSite GATEWAY cassettes, thus simplifying design and construction of the recombinant DNA molecules (Karimi *et al.*, 2007). GATEWAY MultiSite entry clones are potentially adaptable to any model system and an inventory of the entry clones and destination vectors for the GATEWAY MultiSite cloning has been established (Petersen and Stowers, 2011). A new series of binary GATEWAY cloning vectors (pAUL1-20) has been generated for C-terminal and N-terminal proteins fused in-frame to four different tags: a single hemagglutinin epitope, a streptavidin-tagII, both epitopes combined to a double tag, and a triple tag consisting of the double tag extended by a Protein A tag possessing a 3C protease cleavage site (Lyska *et al.*, 2013). For multiple gene expression, modified GATEWAY cloning systems have been developed (Chung *et al.*, 2005; Kimura *et al.*, 2013; Vemanna *et al.*, 2013).

Perspectives

Higher plant transformation technology has become an adaptable platform for cultivar improvement as well as for studying gene functions in plants. Plant DNA can be altered by introducing specific nucleotide substitutions into a gene that change a protein's amino acid sequence, delete genes or chromosomal segments, and insert foreign DNA at precise genomic locations. Such targeted DNA sequence modifications are enabled by sequence-specific nucleases that create double-stranded breaks in the genomic loci. Genomic alterations have recently been achieved through engineered zinc finger nucleases (ZFNs) (Hauschild-Quintern *et al.*, 2012; Tzfira *et al.*, 2012) and transcription activator-like effector nuclease (TALEN)-

type transcription factors (Mussolino and Cathomen, 2012). Through an innovative method, designated directed nuclease editor (DNE), selected genes can be incorporated into the plant genome with an enhanced accuracy (<http://www.precisionbiosciences.com>).

The engineering of a single gene to modify the plant metabolism has appeared promising, but many traits result from many interacting factors that need to be modulated. Such a modulation of complex pathways could be achieved through classical breeding or through the simultaneous engineering of multiple transgenes in nuclear and plastid genomes. It will be interesting to develop versatile molecular toolboxes for the engineering of multiple genes in organelles, such as mitochondria, that hitherto has not been successful. The choice of the preferred technology for multiple transgene engineering will be influenced by many factors, including the T-DNA transfer method, the targeted plant species, the cellular compartmentalization of the pathway of interest, the number of genes to be engineered into the plant, their desired expression levels as well as the available knowledge about metabolite pools, fluxes, and the biochemical regulation of the pathway (Bock, 2013).

Although selectable marker genes are useful tools in the production of transgenic plants by selecting transformed cells from a matrix consisting of mostly untransformed cells, their presence in genetically modified plants and, subsequently, in food, feed, and the environment, are of concern and subject to special governmental regulations in many countries. In addition, they could result in a metabolic burden for the host plants and prevent the reuse of the same selectable markers when a second transformation scheme is needed on the transgenic host. Therefore, innovation in selectable marker removal is necessary to improve existing systems and to develop new technologies. Although the focus of the ZFN and TALEN technologies has been the introduction of local genomic modifications, the ZFN technology has been used for plant selection marker gene deletion. For instance, a preintegrated cassette containing the GUS reporter gene flanked by two ZFN cleavage sites was deleted from a stably transformed plant by crossing it with a second plant expressing a corresponding ZFN gene, a method that can also be applied for selectable marker removal (Petolino *et al.*, 2010). Furthermore, two identical sets of TALEN-binding sequences can be designed to flank a selectable marker in a transformation vector whereby, after expression of TALEN, double-stranded breaks will be induced at both TALEN-binding sequences and remove the selectable marker (Yau and Stewart, 2013).

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References

- ALONSO JM, STEPANOVA AN, LEISSE TJ, KIM CJ, CHEN H, SHINN P, STEVENSON DK, ZIMMERMAN J, BARAJAS P, CHEUK R, *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301: 653-657 [Err. *Science* 301: 1849].
- ANAMI SE, MGUTU AJ, TARACHA C, COUSSENS G, KARIMI M, HILSON P, VAN LIJSEBETTENS M, MACHUKA J (2010) Somatic embryogenesis and plant regeneration of tropical maize genotypes. *Plant Cell Tissue Organ Cult* 102: 285-295.
- ATTAR, LAURENS L, BOUCHERON-DUBUISSON E, GUIVARC'H A, CARNERO E, GIRAUDAT-PAUTOT V, RECH P, CHRQUI D (2009) Pluripotency of *Arabidopsis*

- xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown *in vitro*. *Plant J* 57: 626-644.
- AOYAMA T, DONG C-H, WU Y, CARABELLI M, SESSA G, RUBERTI I, MORELLI G, CHUAN-H (1995) Ectopic expression of the *Arabidopsis* transcriptional activator Athb-1 alters leaf cell fate in tobacco. *Plant Cell* 7: 1773-1785.
- BANNO H, IKEDA Y, NIU Q-W, CHUA N-H (2001) Overexpression of *Arabidopsis* *ESR1* induces initiation of shoot regeneration. *Plant Cell* 13: 2609-2618.
- BAO Y, DHARMAWARDHANAP, MOCKLERTC, STRAUSS SH (2009) Genome scale transcriptome analysis of shoot organogenesis in *Populus*. *BMC Plant Biol* 9: 132.
- BENFEY PN, CHUA N-H (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science* 250: 959-966.
- BEVAN M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* 12: 8711-8721.
- BOCK R (2013) Strategies for metabolic pathway engineering with multiple transgenes. *Plant Mol Biol* 83: 21-31.
- BORKIRD C, CHOI JH, SUNG ZR (1986) Effect of 2,4-dichlorophenoxyacetic acid on the expression of embryogenic program in carrot. *Plant Physiol* 81: 1143-1146.
- BORTESI L, RADEMACHER T, SCHIERMEYER A, SCHUSTER F, PEZZOTTI M, SCHILLBERG S (2012) Development of an optimized tetracycline-inducible expression system to increase the accumulation of interleukin-10 in tobacco BY-2 suspension cells. *BMC Biotechnol* 12: 40.
- BORUC J, VAN DEN DAELE H, HOLLUNDER J, ROMBAUTS S, MYLLE E, HILSON P, INZÉ D, DE VEYLDER L, RUSSINOVA E (2010) Functional modules in the *Arabidopsis* core cell cycle binary protein-protein interaction network. *Plant Cell* 22: 1264-1280.
- BYZOVA M, VERDUYN C, DE BROUWER D, DE BLOCK M (2004) Transforming petals into sepaloid organs in *Arabidopsis* and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner. *Planta* 218: 379-387.
- CHANG AK, DUGGLEBY RG (1998) Herbicide-resistant forms of *Arabidopsis thaliana* acetohydroxyacid synthase: characterization of the catalytic properties and sensitivity to inhibitors of four defined mutants. *Biochem J* 333: 765-777.
- CHANG W-C, LEE T-Y, HUANG H-D, HUANG H-Y, PAN R-L (2008) PlantPAN: Plant promoter analysis navigator, for identifying combinatorial *cis*-regulatory elements with distance constraint in plant gene groups. *BMC Genomics* 9: 561.
- CHATFIELD SP, CAPRON R, SEVERINO A, PENTTILA P-A, ALFRED S, NAHAL H, PROVART NJ (2013) Incipient stem cell niche conversion in tissue culture: using a systems approach to probe early events in *WUSCHEL*-dependent conversion of lateral root primordia into shoot meristems. *Plant J* 73: 798-813.
- CHE P, LALL S, HOWELL SH (2007) Developmental steps in acquiring competence for shoot development in *Arabidopsis* tissue culture. *Planta* 226: 1183-1194.
- CHE P, LALL S, NETTLETON D, HOWELL SH (2006) Gene expression programs during shoot, root, and callus development in *Arabidopsis* tissue culture. *Plant Physiol* 141: 620-637.
- CHEO DL, TITUS SA, BYRD DRN, HARTLEY JL, TEMPLE GF, BRASCH MA (2004) Concerted assembly and cloning of multiple DNA segments using *in vitro* site-specific recombination: functional analysis of multi-segment expression clones. *Genome Res* 14: 2111-2120.
- CHRISTENSEN AH, SHARROCK RA, QUAIL PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol* 18: 675-689.
- CHRISTIANSON ML, WARNICK DA (1983) Competence and determination in the process of *in vitro* shoot organogenesis. *Dev Biol* 95: 288-293.
- CHU H, JEONG JC, KIM W-J, CHUNG DM, JEON HK, AHN YO, KIM SH, LEE H-S, KWAK SS, KIM CY (2013) Expression of the sweetpotato R2R3 type *lbMYB1a* gene induces anthocyanin accumulation in *Arabidopsis*. *Physiol Plant* 148: 189-199.
- CHUNG S-M, FRANKMAN EL, TZFIRAT (2005) A versatile vector system for multiple gene expression in plants. *Trends Plant Sci* 10: 357-361.
- CLOUGH SJ, BENT AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16: 735-743.
- COUSSENS G, AESAERT S, VERELST W, DEMEULENAERE M, DE BUCK S, NUJGUNA E, INZÉ D, VAN LIJSEBETTENS M (2012) *Brachypodium distachyon* promoters as efficient building blocks for transgenic research in maize. *J Exp Bot* 63: 4263-4273.
- DATTA SK, PETERHANS A, DATTA K, POTRYKUS I (1990) Genetically engineered fertile indica-rice recovered from protoplasts. *Biotechnology* 8: 736-740.
- DE BLOCK M, BOTTERMAN J, VANDEWIELE M, DOCKX J, THOEN C, GOSSELÉ V, MOVVA R, THOMPSON C, VAN MONTAGU M, LEEMANS J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J* 6: 2513-2518.
- DE BLOCK M, DE SONVILLE A, DE BROUWER D (1995) The selection mechanism of phosphinothricin is influenced by the metabolic status of the tissue. *Planta* 197: 619-626.
- DE BLOCK M, HERRERA-ESTRELLA L, VAN MONTAGU M, SCHELL J (1984) Expression of foreign genes in regenerated plants and their progeny. *EMBO J* 3: 1681-1689.
- DE CLEENE M, DE LEY J (1976) The host range of crown gall. *Bot Rev* 42: 389-466.
- DUCLERCQ J, SANGWAN-NORREEL B, CATTEROU M, SANGWAN RS (2011) *De novo* shoot organogenesis: from art to science. *Trends Plant Sci* 16: 597-606.
- DUGDALE B, MORTIMER CL, KATO M, JAMES TA, HARDING RM, DALE JL (2013) In plant activation: an inducible, hyperexpression platform for recombinant protein production in plants. *Plant Cell* 25: 2429-2443.
- ELHITI M, STASOLLAC (2012) *In vitro* shoot organogenesis and hormone response are affected by the altered levels of *Brassica napus* meristem genes. *Plant Sci* 190: 40-51.
- FRALEY RT, ROGERS SG, HORSCH RB, SANDERS PR, FLICK JS, ADAMS SP, BITTNER ML, BRAND LA, FINK CL, FRY JS, GALLUPPI GR, GOLDBERG SB, HOFFMANN NL, WOO SC (1983) Expression of bacterial genes in plant cells. *Proc Natl Acad Sci USA* 80: 4803-4807.
- FRAME BR, SHOU H, CHIKWAMBA RK, ZHANG Z, XIANG C, FONGER TM, PEGG SEK, LI B, NETTLETON DS, PEI D, WANG K (2002) *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol* 129: 13-22.
- FROMM ME, MORRISH F, ARMSTRONG C, WILLIAMS R, THOMAS J, KLEIN TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Biotechnology* 8: 833-839.
- GAO X, ZHANG L, ZHOU S, WANG C, DENG X, ZHANG H, YANG G, JAVEED H, HE G (2011). *AtMYB12* gene: a novel visible marker for wheat transformation. *Mol Biol Rep* 38: 183-190.
- GELVIN SB (2012) Traversing the Cell: *Agrobacterium* T-DNA's journey to the host genome. *Front Plant Sci* 3: 52.
- GHEYSEN G, VILLARROEL R, VAN MONTAGU M (1991) Illegitimate recombination in plants: a model for T-DNA integration. *Genes Dev* 5: 287-297.
- GOLDSWORTHY A, STREET HE (1965) The carbohydrate nutrition of tomato roots. VIII. The mechanism of the inhibition by D-mannose of the respiration of excised roots. *Ann Bot* 29: 45-58.
- HARTLEY JL, TEMPLE GF, BRASCH MA (2000) DNA cloning *in vitro* using site-specific recombination. *Genome Res* 10: 1788-1795.
- HASELOFF J, SIEMERING KR, PRASHER DC, HODGE S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc Natl Acad Sci USA* 94: 2122-2127.
- HAUSCHILD-QUINTERN J, PETERSEN B, COST GJ, NIEMANN H (2013) Gene knockout and knockin by zinc-finger nucleases: current status and perspectives. *Cell Mol Life Sci* 70: 2969-2983.
- HERRERA-ESTRELLA L, DE BLOCK M, MESSENS E, HERNALSTEENS J-P, VAN MONTAGU M, SCHELL J (1983) Chimeric genes as dominant selectable markers in plant cells. *EMBO J* 2: 987-995.
- HIMMELBACH A, ZIEROLF U, HENSEL G, RIECHEN J, DOUCHKOV D, SCHWEIZER P, KUMLEHN J (2007) A set of modular binary vectors for transformation of cereals. *Plant Physiol* 145: 1192-1200.
- HOOD EE, HELMER GL, FRALEY RT, CHILTON M-D (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J Bacteriol* 168: 1291-1301.
- HORSCH RB, FRALEY RT, ROGERS SG, SANDERS PR, LLOYD A, HOFFMANN N (1984) Inheritance of functional foreign genes in plants. *Science* 223: 496-498.
- JEFFERSON RA, KAVANAGH TA, BEVAN MW (1987) GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6: 3901-3907.
- JIANG C, MITHANI A, GAN X, BELFIELD EJ, KLINGLER JP, ZHU J-K, RAGOUS-

- SIS J, MOTT R, HARBERD NP (2011) Regenerant *Arabidopsis* lineages display a distinct genome-wide spectrum of mutations conferring variant phenotypes. *Current Biol* 21:1385-1390.
- JOERSBO M, DONALDSON I, KREIBERG J, PETERSEN SG, BRUNSTEDT J, OKKELS FT (1998) Analysis of mannose selection used for transformation of sugar beet. *Mol Breed* 4: 111-117.
- KARIMI M, DEPICKER A, HILSON P (2007) Recombinational cloning with plant Gateway vectors. *Plant Physiol* 145: 1144-1154.
- KARIMI M, INZÉ D, VAN LIJSEBETTENS M, HILSON P (2013) Gateway vectors for transformation of cereals. *Trends Plant Sci* 18: 1-4.
- KAWAHIGASHI H, HIROSE S, IWAI T, OHASHI Y, SAKAMOTO W, MAEKAWA M, OHKAWA Y (2007). Chemically induced expression of rice *OSB2* under the control of the *OsPR1.1* promoter confers increased anthocyanin accumulation in transgenic rice. *J Agric Food Chem* 55: 1241-1247.
- KIKUCHI A, SANUKI N, HIGASHI K, KOSHIBA T, KAMADA H (2006) Abscisic acid and stress treatment are essential for the acquisition of embryogenic competence by carrot somatic cells. *Planta* 223: 637-645.
- KIMURA T, NAKAO A, MURATA S, KOBAYASHI Y, TANAKA Y, SHIBAHARA K, KAWAZU T, NAKAGAWAT (2013) Development of the Gateway recycling cloning system for multiple linking of expression cassettes in a defined order, and direction on Gateway compatible binary vectors. *Biosci Biotechnol Biochem* 77: 430-434.
- KIRIENKO DR, LUO A, SYLVESTER AW (2012) Reliable transient transformation of intact maize leaf cells for functional genomics and experimental study. *Plant Physiol* 159: 1309-1318.
- KLEIN TM, FROMM M, WEISSINGER A, TOMES D, SCHAAF S, SLETTEN M, SANFORD JC (1988a) Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. *Proc Natl Acad Sci USA* 85: 4305-4309.
- KOMORI T, IMAIYAMA T, KATO N, ISHIDA Y, UEKI J, KOMARI T (2007) Current status of binary vectors and superbinary vectors. *Plant Physiol* 145: 1155-1160.
- KOORNNEEF M, BADE J, HANHART C, HORSMAN K, SCHEL J, SOPPE W, VERKERK R, ZABEL P (1993) Characterization and mapping of a gene controlling shoot regeneration in tomato. *Plant J* 3: 131-141.
- KRAMER C, DIMAIO J, CARSWELL GK, SHILLITO RD (1993) Selection of transformed protoplast-derived *Zea mays* colonies with phosphinothricin and a novel assay using the pH indicator chlorophenol red. *Planta* 190: 454-458.
- LEE L-Y, GELVIN SB (2008) T-DNA binary vectors and systems. *Plant Physiol* 146: 325-332.
- LESCOT M, DÉHAIS P, THIJS G, MARCHAL K, MOREAU Y, VAN de PEER Y, ROUZÉ P, ROMBAUTS S (2002) PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucleic Acids Res* 30: 325-327.
- LLOYD AM, WALBOT V, DAVIS RW (1992). *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators *R* and *C1*. *Science* 258: 1773-1775.
- LLOYD AM, SCHENA M, WALBOT V, DAVIS RW (1994) Epidermal cell fate determination in *Arabidopsis*: patterns defined by a steroid-inducible regulator. *Science* 266: 436-439.
- LOTAN T, OHTO MA, YEE KM, WEST MAL, LO R, KWONG RW, YAMAGISHI K, FISCHER RL, GOLDBERG RB, HARADA JJ (1998) *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell* 93: 1195-1205.
- LUDWIG SR, BOWEN B, BEACH L, WESSLER SR (1990). A regulatory gene as a novel visible marker for maize transformation. *Science* 247: 449-450.
- LYSKAD, ENGELMANN K, MEIERHOFF K, WESTHOFF P (2013) pAUL: A Gateway-based vector system for adaptive expression and flexible tagging of proteins in *Arabidopsis*. *PLoS ONE* 8: e53787.
- MANN DGJ, KING ZR, LIU W, JOYCE BL, PERCIFIELD RJ, HAWKINS JS, LAFAYETTE PR, ARTELT BJ, BURRIS JN, MAZAREI M, BENNETZEN JL, PARROTT WA, STEWART CN. Jr. (2011) Switchgrass (*Panicum virgatum* L.) polyubiquitin gene (*PvUbi1* and *PvUbi2*) promoters for use in plant transformation. *BMC Biotechnol* 11: 74.
- MANN DGJ, LAFAYETTE PR, ABERCROMBIE LL, KING ZR, MAZAREI M, HALTER MC, POOVAIAH CR, BAXTER H, SHEN H, DIXON RA, PARROTT WA, STEWART CN. Jr. (2012) Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species. *Plant Biotechnol J* 10: 226-236.
- MARIANI C, DE BEUCKELEER M, TRUETTNER J, LEEMANS J, GOLDBERG RB (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene. *Nature* 347: 737-741.
- McELROY D, ZHANG W, CAO J, WU R (1990) Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* 2: 163-171.
- MIKI B, McHUGH S (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *J Biotechnol* 107: 193-232.
- MILLAR AJ, SHORT SR, HIRATSUKA K, CHUA N-H, KAY SA (1992) Firefly luciferase as a reporter of regulated gene expression in higher plants. *Plant Mol Biol Rep* 10: 324-337.
- MURAKAMI T, ANZAI H, IMAI S, SATOH A, NAGAOKA K, THOMPSON CJ (1986) The bialaphos biosynthetic genes of *Streptomyces hygrosopicus*: molecular cloning and characterization of the gene cluster. *Mol Gen Genet* 205: 42-50.
- MURANAKAT (2011) Plant Gateway vectors for RNAi as a tool for functional genomic studies. *Methods Mol Biol* 744: 27-35.
- MUSSOLINO C, CATHOMEN T (2012) TALE nucleases: tailored genome engineering made easy. *Curr Opin Biotechnol* 23: 644-650.
- NAGEL RJ, MANNERS JM, BIRCH RG (1992) Evaluation of an ELISA assay for rapid detection and quantification of neomycin phosphotransferase II in transgenic plants. *Plant Mol Biol Rep* 10: 263-272.
- NAGY JI, MALIGA P (1976) Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. *Z Pflanzenphysiol* 78: 453-455.
- NAQVI S, ZHU C, FARRE G, RAMESSAR K, BASSIE L, BREITENBACH J, PEREZ CONESA D, ROS G, SANDMANN G, CAPELL T, CHRISTOU P (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proc Natl Acad Sci USA* 106: 7762-7767.
- NIEDZ RP, SUSSMAN MR, SATTERLEE JS (1995) Green fluorescent protein: an *in vivo* reporter of plant gene expression. *Plant Cell Rep* 14: 403-406.
- O'KENNEDY MM, STARK HC, DUBE N (2011) Biolistic-mediated transformation protocols for maize and pearl millet using pre-cultured immature zygotic embryos and embryogenic tissue. *Methods Mol Biol* 710: 343-354.
- ODELL JT, NAGY F, CHUA N-H (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 313: 810-812.
- OLSZEWSKI NE, MARTIN FB, AUSUBEL FM (1988) Specialized binary vector for plant transformation: expression of the *Arabidopsis thaliana* AHAS gene in *Nicotiana tabacum*. *Nucleic Acids Res* 16: 10765-10782.
- OW DW, WOOD KV, DeLUCA M, de WET JR, HELINSKI DR, HOWELL SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* 234: 856-859.
- PADIDAM M (2003) Chemically regulated gene expression in plants. *Curr Opin Plant Biol* 6: 169-177.
- PARCY F, VALON C, RAYNAL M, GAUBIER-COMELLA P, DELSENY M, GIRAUDAT J (1994) Regulation of gene expression programs during *Arabidopsis* seed development: roles of the *ABI3* locus and of endogenous abscisic acid. *Plant Cell* 6: 1567-1582.
- PETERSEN LK, STOWERS RS (2011) A Gateway multisite recombination cloning toolkit. *PLoS ONE* 6: e24531.
- PETOLINO JF, WORDEN A, CURLEE K, CONNELL J, STRANGE MOYNAHAN TL, LARSEN C, RUSSELL S (2010) Zinc finger nuclease-mediated transgene deletion. *Plant Mol Biol* 73: 617-628.
- PRAKASH AP, KUMAR PP (2002) *PkMADS1* is a novel MADS box gene regulating adventitious shoot induction and vegetative shoot development in *Paulownia kawakamii*. *Plant J* 29: 141-151.
- PRASHER DC, ECKENRODE VK, WARD WW, PRENDERGAST FG, CORMIER MJ (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111: 229-233.
- REED J, PRIVALLE L, POWELL ML, MEGHJI M, DAWSON J, DUNDER E, SUTTIE J, WENCK A, LAUNIS K, KRAMER C, CHANG Y-F, HANSEN G, WRIGHT M (2001) Phosphomannose isomerase: an efficient selectable marker for plant transformation. *In Vitro Cell Dev Biol-Plant* 37: 127-132.
- REISS B, SPRENGEL R, WILL H, SCHALLER H (1984) A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. *Gene* 30: 211-218.
- ROSELLINI D (2011) Selectable marker genes from plants: reliability and potential. *In Vitro Cell Dev Biol-Plant* 47: 222-233.

- ROSLAN HA, SALTER MG, WOOD CD, WHITE MRH, CROFT KP, ROBSON F, COUPLAND G, DOONAN J, LAUFS P, TOMSETT AB, CADDICK MX (2001) Characterization of the ethanol-inducible *alc* gene-expression system in *Arabidopsis thaliana*. *Plant J* 28: 225-235.
- SCHMIDT EDL, GUZZO F, TOONEN MAJ, de VRIES S (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development* 124: 2049-2062.
- SEOM-S, JIN M, LEE S-S, KWONS-J, MUN J-H, PARK B-S, VISSER RGF, BONNEMA G, SOHN S-H (2013) Mapping quantitative trait loci for tissue culture response in VCS3M-DH population of *Brassica rapa*. *Plant Cell Rep* 32: 1251-1261.
- SHEEN J (2001) Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. *Plant Physiol* 127: 1466-1475.
- SHIMOMURA O, JOHNSON FH, SAIGA Y (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusa, *Aequorea*. *J Cell Comp Physiol* 59: 223-239.
- SHOU H, FRAME BR, WHITHAM SA, WANG K (2004) Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. *Mol Breed* 13: 201-208.
- SHRAWAT AK, LÖRZ H (2006) *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. *Plant Biotechnol J* 4: 575-603.
- SKOOG F, MILLER CO (1957) Chemical regulation of growth and organ formation in plant tissues cultures *in vitro*. *Symp Soc Exp Biol* 11: 118-131.
- SMIRNOVA OG, IBRAGIMOVASS, KOCHETOV AV (2012) Simple database to select promoters for plant transgenesis. *Transgenic Res* 21: 429-437.
- STEWART FC, MAPES MO, MEARS K (1958) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *Am J Bot* 45: 705-708.
- SUGIMOTO K, GORDON SP, MEYEROWITZ EM (2011) Regeneration in plants and animals: dedifferentiation, transdifferentiation, or just differentiation? *Trends Cell Biol* 21: 212-218.
- SUGIMOTO K, JIAO Y, MEYEROWITZ EM (2010) *Arabidopsis* regeneration from multiple tissues occurs via a root development pathway. *Dev Cell* 18: 463-471.
- SUGIYAMA M (1999) Organogenesis *in vitro*. *Curr Opin Plant Biol* 2: 61-64.
- THOMPSON CJ, RAOMOVVA N, TIZARD R, CRAMER R, DAVIES JE, LAUWEREYS M, BOTTERMAN J (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *EMBO J* 6: 2519-2523.
- TORELLI A, BORINATO M, SORAGNI E, BOLPAGNI R, BOTTURA C, BRANCA C (2004) The delay in hormonal treatment modulates the expression of *LESK1*, a gene encoding a putative serine-threonine kinase, marker of *in vitro* caulogenesis in tomato (*Lycopersicon esculentum* Mill.). *Plant Sci* 167: 607-620.
- TRUJILLO-MOYA C, GISBERT C (2012) The influence of ethylene and ethylene modulators on shoot organogenesis in tomato. *Plant Cell Tissue Organ Cult* 111: 41-48.
- TZFIRA T, CITOVSKY V (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Curr Opin Biotechnol* 17: 147-154.
- TZFIRA T, WEINTHAL D, MARTON I, ZEEVI V, ZUKER A, VAINSTEIN A (2012) Genome modifications in plant cells by custom-made restriction enzymes. *Plant Biotechnol J* 10: 373-389.
- VALVEKENS D, VAN MONTAGU M, VAN LIJSEBETTENS M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc Natl Acad Sci USA* 85: 5536-5540.
- van den ELZEN PJM, TOWNSEND J, LEE KY, BEDBROOK JR (1985) A chimaeric hygromycin resistance gene as a selectable marker in plant cells. *Plant Mol Biol* 5: 299-302.
- VAN LIJSEBETTENS M, VANDERHAEGHEN R, VAN MONTAGU M (1991) Insertional mutagenesis in *Arabidopsis thaliana*: isolation of a T-DNA-linked mutation that alters leaf morphology. *Theor Appl Genet* 81: 277-284.
- VEMANNA RS, CHANDRASHEKAR BK, HANUMANTHA RAO HM, SATHYANARAYANAGUPTA SK, SARANGI KS, NATARAJA KN, UDAYAKUMAR M (2013) A modified MultiSite Gateway cloning strategy for consolidation of genes in plants. *Mol Biotechnol* 53: 129-138.
- VOSS, U., LARRIEU, A and WELLS, D.M. (2013). From jellyfish to biosensors: the use of fluorescent proteins in plants. *Int. J. Dev. Biol.* 57: 525-533. [DOI: 10.1387/ijdb.130208dw]
- WALDRON C, MURPHY EB, ROBERTS JL, GUSTAFSON GD, ARMOUR SL, MALCOLM SK (1985) Resistance to hygromycin B: a new marker for plant transformation studies. *Plant Mol Biol* 5: 103-108.
- WOHLLEBEN W, ARNOLD W, BROER I, HILLEMANN D, STRAUCH E, PÜHLER A (1988) Nucleotide sequence of the phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. *Gene* 70: 25-37.
- XU X, XIE Q, McCLUNG CR (2010) Robust circadian rhythms of gene expression in *Brassica rapa* tissue culture. *Plant Physiol* 153: 841-850.
- YANG M, BARANOV E, MOOSSA AR, PENMAN S, HOFFMAN RM (2000) Visualizing gene expression by whole-body fluorescence imaging. *Proc Natl Acad Sci USA* 97: 12278-12282.
- YANG T-T, CHENG L, KAIN SR (1996) Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucleic Acids Res* 24: 4592-4593.
- YAO J-L, TOMES S, GLEAVE AP (2013) Transformation of apple (*Malus x domestica*) using mutants of apple *acetolactate synthase* as a selectable marker and analysis of the T-DNA integration sites. *Plant Cell Rep* 32: 703-714.
- YAU Y-Y, STEWART CN Jr (2013) Less is more: strategies to remove marker genes from transgenic plants. *BMC Biotechnol* 13: 36.
- YILMAZ A, NISHIYAMA MY Jr, GARCIA FUENTES B, MENDES SOUZA G, JANIES D, GRAY J, GROTEWOLD E (2009) GRASSIUS: a platform for comparative regulatory genomics across the grasses. *Plant Physiol* 149: 171-180.
- ZIMMERMAN JL (1993) Somatic embryogenesis: a model for early development in higher plants. *Plant Cell* 5: 1411-1423.

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